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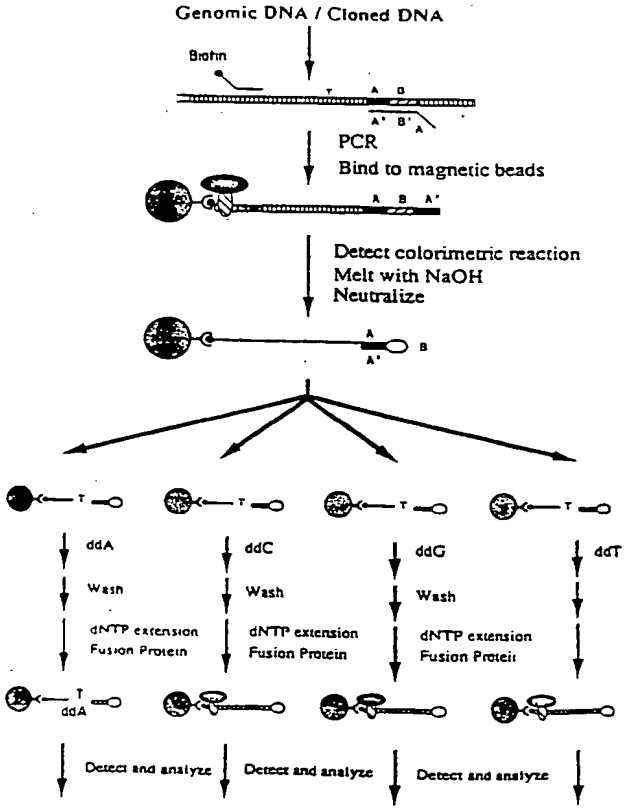
(57) Abstract

The invention provides a method of introducing a 3'-terminal loop structure onto a target sequence of one strand of double stranded DNA, said target sequence having a region A at the 3'-terminus thereof and there being optionally a DNA region B which extends 3' from region A, whereby said double-stranded DNA is subjected to polymerase chain reaction (PCR) amplification using a first primer hybridising to the 3'-terminus of the sequence complementary to the target sequence, which first primer is immobilised or provided with means for attachement to a solid support, and a second primer having a 3'-terminal sequence which hybridises to at least a portion of A and/or B of the target sequence while having at its 5'-end a sequence substantially identical to A, said amplification producing double-stranded target DNA having at the 3'-end of the target sequence, in the following order, the region A, a region capable of forming a loop and a sequence A' complementary to sequence A, whereafter the amplified double-stranded DNA is subjected in immobilised form to strand separation whereby the non-immobilised target strand is liberated and region A' is permitted or caused to hybridise to region A, thereby forming said loop.



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Loop Structures

This invention relates to loop structures at the 3'-terminal of a single strand of DNA.

It is known in molecular biology that single stranded nucleic acids are known to fold back on themselves and form loop structures and short double stranded segments held together by hydrogen bonding.

Such secondary structures can affect the susceptibility of the nucleic acid to interaction with enzymes, e.g. polymerases, hydrolases and such like. In some instances the formation of loop structures by single stranded nucleic acid will compete with the hybridisation of a probe or a primer to a target sequence of the nucleic acid. In the case where a primer is being used, e.g. for sequencing DNA, the formation of loops at or near the sequence at which the primer hybridises will lower the efficiency of hybridisation and thereby effect the clarity of sequencing results.

The present invention is based on the concept of using PCR to introduce loop structures which provide a primer at the 3'-terminal of a DNA strand of interest.

DNA molecules are often present in samples in small quantities and in order to amplify such DNA, the polymerase chain reaction (PCR) method has been developed. In this technique a pair of polymerisation primers specific to known sequences of the target DNA are selected, one hybridising at or near the 5' end of one of the strands and the other at or near the 5' end of the complementary strand such that in the presence of a polymerase, each primer produces a DNA sequence extending the full length of the target DNA template. If the DNA so produced is then subjected to strand separation, typically by melting at a temperature of

about 90°C, the newly formed single stranded DNA sequences will hybridise to excess primer present in the mixture, usually after reducing the temperature to the range suitable for annealing, whereupon in the presence of the polymerase, further DNA strands are synthesised, this time extending only between the termini of the two primers. The polymerase is preferably capable of surviving the high temperature used in the strand separation step, a suitable thermophilic polymerase, namely Taq, having recently become available. If an excess of the two primers and of nucleotides needed for DNA synthesis is maintained in the medium, it is possible to operate a repeated cyclic process in which the separate strands are synthesised, separated, annealed to primer and new strands synthesised, merely by raising and lowering the temperature between the optimal temperatures for each of the above stages. In this way, it is found that amplification of the original target DNA can be exponential and million-fold increases of concentration can be effected in a relatively short time. Such amplified DNA may then be sequenced or otherwise investigated.

The invention provides a method of introducing a 3'-terminal loop structure onto a target sequence of one strand of double stranded DNA, said target sequence having a region A at the 3'-terminus thereof and there being optionally a DNA region B which extends 3' from region A, whereby said double-stranded DNA is subjected to polymerase chain reaction (PCR) amplification using a first primer hybridising to the 3'-terminus of the sequence complementary to the target sequence, which first primer is immobilised or provided with means for attachment to a solid support, and a second primer having a 3'-terminal sequence which hybridises to at least a portion of A and/or B of the target sequence while having at its 5'-end a sequence substantially identical to A, said amplification producing double-

stranded target DNA having at the 3'-end of the target sequence, in the following order, the region A, a region capable of forming a loop and a sequence A' complementary to sequence A, whereafter the amplified double-stranded DNA is subjected in immobilised form to strand separation whereby the non-immobilised target strand is liberated and region A' is permitted or caused to hybridise to region A, thereby forming said loop.

It will be understood that the region capable of forming a loop comprises all or part of region B (if present) and/or a sequence complementary to an optional loop-forming linker sequence in the second primer between region A' and A. If the second primer hybridises to region A on the target sequence it will be highly desirable that a loop-forming linker sequence is present in the primer in order to allow substantially full hybridisation of A' to A in loop formation. If the second primer hybridises to a part of region B remote from region A on the target sequence, the the portion of B between region A and the region of hybridisation will form the loop together with the region of hybridisation and any loop-forming linker sequence (if present) in the primer.

Although the second primer has a sequence A'-B (if present) and/or a loop-forming linker sequence-A and there is the possibility of the primer looping backing on itself (rather than acting as a primer), this possibility of "self priming" is avoided by selecting a higher annealing temperature than that at which the primer preferentially anneals to itself.

It will be appreciated that both the immobilised strand and the non-immobilised strand will be capable of forming loop structures. However, only the immobilised strand will have the sequence A' in the right orientation to serve as a primer for chain extension. Further, it will be noted that the convention of reading sequences in a 5' to 3' direction has been followed.

Thus the sequence of the primer substantially identical to A is substantially identical reading in a 5' to 3' direction.

An advantage of this invention is that a primer is incorporated into the 3' end of a strand of DNA and the primer can then be used, for example, in sequencing the strand or other procedures. It is clear that each immobilised template will have a built-in primer and that the primer will be kept in relatively close proximity to the region where it hybridises by virtue of the linker sequence. Thus, even if conditions are varied such that strand separation occurs, the primer, being joined to the template, will readily re-hybridise.

If desired, it is possible to form an restriction endonuclease (RE) site close to the loop so that it may be removed, e.g. to allow strand separation during sequencing. This arrangement will be desirable, for example when the sequencing is by the Sanger method (e.g. Sanger F. et al (1977) PNAS (USA) 74: 5463-5467) and lengths of newly formed dideoxynucleotide terminated strands need to be separated from target DNA which has served as a template. Suitable RE sites and their mode of incorporation into a primer will be known to the skilled worker and are taught by the literature, for example Molecular Cloning: a laboratory manual by T. Maniatis et al. For example, a linker sequence in the second primer may contain complementary palindromic sequences adjacent A and A' respectively so that on loop formation these adjacent regions hybridise to form an RE site in the double stranded portion which site allows for removal of the loop. Alternatively, region A A' may be chosen such that it contains an RE site.

Advantageously the present invention can be combined with the invention taught in our co-pending application of even date entitled "Chemical Method" (Agents ref.: 75.57465).

Our co-pending case is based on the concept of

amplifying and then immobilising the DNA of interest followed by a polymerase reaction carried out on four aliquots of the immobilised DNA in single stranded form. Each aliquot uses the same specific extension primer and a different dideoxynucleotide but no deoxynucleotides so that only the dideoxynucleotide complementary to the base in the target position is incorporated; the target position being directly adjacent to the 3' end of the specific extension primer hybridising to the DNA. Put another way, the target position on the immobilised strand is immediately 5' of where the extension primer hybridises to the DNA. Chain extension using normal deoxynucleotides is then effected using the specific primer so that the dideoxy-blocked DNA will remain unreacted while the un-blocked DNA will form double stranded DNA. Various methods may then be used to distinguish double stranded DNA from non-extended, i.e. substantially single stranded DNA, and thus enable the base in the target position to be identified. Preferably, the DNA of interest is amplified by PCR.

A primer is normally added to each of the aliquots for the dideoxy and extension reactions. However, using the present invention, the target DNA may be provided with a loop-attached primer which, as indicated above is stable to variations in conditions and substantially reduces or eliminates losses in passing from the dideoxy reaction to chain extension.

Specifically the invention taught in our co-pending application may be modified in accordance with the present invention so that sample DNA is provided with a loop-attached 3' primer which hybridises to the immobilised DNA immediately adjacent to the target position; each of four aliquots of the immobilised single stranded DNA is then subjected to a polymerase reaction in the presence of a dideoxynucleotide, each aliquot using a different dideoxynucleotide whereby only the dideoxynucleotide complementary to the base in the

target position becomes incorporated; the four aliquots are then subjected to extension in the presence of all four deoxynucleotides, whereby in each aliquot the DNA which has not reacted with the dideoxynucleotide is extended to form double stranded DNA while the dideoxy-blocked DNA remains as non-extended stranded DNA; followed by identification of the double stranded and/or non-extended DNA to indicate which dideoxynucleotide was incorporated and hence which base was present in the target position.

It is desirable that the effectiveness of the PCR is assessed, e.g. to determine whether or not sufficient DNA has been formed to give clear results with a relatively low level of background. Various tests are known in the art but we prefer to use the solid phase approach we described earlier for detection of immobilized amplified nucleic acids, designated DIANA (PCT/EP90/00454), which has been used for example in its preferred embodiment in the colorimetric detection of in vitro amplified DNA. The assay is based on the use of a biotinylated or otherwise functionalised PCR primer, which is used to capture in vitro amplified material on, for example, streptavidin-coated magnetic beads. The other PCR primer contains a "handle", such as a lac operator sequence, allowing colorimetric detection of the captured DNA using a LacI repressor-β-galactosidase fusion protein. (Wahlberg, J., Lundeberg, J., Hultman, T. and Uhlén, M. (1990) "General colorimetric method for DNA diagnostics allowing direct solid-phase genomic sequencing of the positive samples." Proc. Natl. Acad. Sci U.S.A. 87, 6569-6573). The preferred form of the qualitative DIANA assay combines the advantages of the PCR method with the high specificity and stability of the biotin-streptavidin system and the simplicity of a colorimetric detection based on β-galactosidase. The strong interaction between biotin and streptavidin ($K_d=10^{-15}$ M^{-1}) accentuates the efficiency of the system.

The magnetic beads as solid support ensure that no centrifugations, filtrations or precipitations are needed (T. Hultman, S. Ståhl, E. Hornes and M. Uhlén Nucl. Acids Res. 17, 4937 (1989)).

A number of proteins are known which bind to specific DNA sequences and are often involved in genetic processes such as switching operons on and off. One such protein is the lac repressor LacI which reacts with the lac operator (lacOP) to inhibit transcription. Thus, if the recognition site is the DNA sequence lacOP, the label can be attached via the protein LacI. It is particularly convenient to devise a fusion protein of a DNA binding protein such as LacI with a further protein which can be subsequently used for detection for example using methods based on colour fluorescence or chemiluminescence. Examples of such proteins are β-galactosidase, alkaline phosphatase and peroxidase.

It is preferred to use as a label a LacI repressor-β-galactosidase fusion protein which recognises a 21 base pair lac operator sequence introduced at the end of the amplified DNA, by one of the primers, preferably the immobilised primer. The fusion protein will bind to the lac OP sequence of the DNA and the addition of ONPG (ortho-nitrophenyl-β-D-galactoside) will lead to a colour formation which can be assessed spectrophotometrically. Use of this fusion protein and ONPG allows for a fast simple colorimetric assay which does not have the safety problems associated with using radiolabels. IPTG (n-isopropyl-β-D-thiogalactopyranoside), for example, can be added to release the fusion protein from the DNA.

The specificity of the process is greatly increased by including a first-stage PCR amplification step. By such preliminary amplification, the concentration of target DNA is greatly increased with respect to other DNA which may be present in the sample and a second-stage amplification with at least one primer specific to

a different sequence of the target DNA, as described in PCT/EP 90/00454, significantly enhances the signal due to the target DNA relative to the 'background noise'.

Two-stage PCR (using nested primers), as described in our co-pending application PCT/EP90/00454, may be used to enhance the signal to noise ratio and thereby increase the sensitivity of the method according to the invention.

Immobilisation of the amplified DNA may either take place as part of the PCR amplification itself, as where one or more primers are attached to a support, or alternatively one or more of the primers may carry a functional group permitting subsequent immobilisation, eg. a biotin or thiol group. Immobilisation by the 5' end of the primer allows the strand of DNA emanating from that primer to be attached to a solid support and have its 3' end remote from the support and available for subsequent chain extension by polymerase.

The solid support may conveniently take the form of microtitre wells, which are advantageously in the conventional 8 x 12 format, or dipsticks which may be made of polystyrene activated to bind the primer DNA (K Almer, Doctoral Theses, Royal Institute of Technology, Stockholm, Sweden, 1988). The support may also comprise particles, fibres or capillaries made, for example, of agarose, cellulose, alginate, Teflon or polystyrene. The support may also comprise magnetic particles eg the superparamagnetic beads produced by Dynal AS (Oslo, Norway).

The solid support may carry functional groups such as hydroxyl, carboxyl, aldehyde or amino groups, or other moieties such as avidin or streptavidin, for the attachment of primers. These may in general be provided by treating the support to provide a surface coating of a polymer carrying one of such functional groups, e.g. polyurethane together with a polyglycol to provide hydroxyl groups, or a cellulose derivative to provide

hydroxyl groups, a polymer or copolymer of acrylic acid or methacrylic acid to provide carboxyl groups or an aminoalkylated polymer to provide amino groups. US Patent No. 4654267 describes the introduction of many such surface coatings.

Any suitable polymerase may be used, although it is preferred to use a thermophilic enzyme such as Taq polymerase to permit the repeated temperature cycling without having to add further polymerase, e.g. Klenow fragment, in each cycle.

The target DNA may be cDNA synthesised from RNA in the sample and the method of the invention is thus applicable to diagnosis on the basis of characteristic RNA. Such preliminary synthesis can be carried out by a preliminary treatment with a reverse transcriptase, conveniently in the same system of buffers and bases to be used in the subsequent PCR steps. Since the PCR procedure requires heating to effect strand separation, the reverse transcriptase will be inactivated in the first PCR cycle. When mRNA is the sample nucleic acid, it may be advantageous to submit the initial sample, e.g. a serum sample, to treatment with an immobilised polydT oligonucleotide in order to retrieve all mRNA via the terminal polyA sequences thereof. Alternatively, a specific oligonucleotide sequence may be used to retrieve the RNA via a specific RNA sequence. The oligonucleotide can then serve as a primer for cDNA synthesis, as described in International Patent Application PCT/89EP/00304.

PCR has been discussed above as a preferred method of initially amplifying target DNA although the skilled person will appreciate that other methods may be used instead of in combination with PCR. A recent development in amplification techniques which does not require temperature cycling or use of a thermostable polymerase is Self Sustained Sequence Replication (3SR). 3SR is modelled on retroviral replication and may be

used for amplification (see for example Gingeras, T.R. et al PNAS (USA) 87:1874-1878 and Gingeras, T.R. et al PCR Methods and Applications VOL. 1, PP 25-33).

Advantageously, the primers are sufficiently large to provide appropriate levels of hybridisation, yet still reasonably short in order to avoid unnecessary chemical synthesis. It will be clear to persons skilled in the art that the size of the primers and the stability of hybridisation will be dependent to some degree on the ratio of A-T to C-G base pairings, since more hydrogen bonding is available in a C-G pairing. Also, the skilled person will consider the degree of homology between the extension primer to other parts of the amplified sequence and choose the degree of stringency accordingly. Guidance for such routine experimentation can be found in the literature, for example, Molecular Cloning: a laboratory manual by Sambrook, J., Fritsch, E.F., and Maniatis, T (1989).

The polymerase reaction in the presence of dideoxy nucleotides is carried out using a polymerase which will incorporate dideoxynucleotides, e.g. T7 polymerase, Klenow or Sequenase Ver. 2.0 (USB U.S.A.). However, it is known that many polymerases have a proof-reading or error checking ability and that 3' ends available for chain extension are sometimes digested by one or more nucleotides. If such digestion occurs in the method according to the invention the level of background noise increases. In order to avoid this problem it is desirable to add to each aliquot fluoride ions or nucleotide monophosphates which suppress 3' digestion by polymerase.

Identification of the double stranded and/or non-extended DNA is possible via a variety of means. With regard to the double stranded DNA, conventional techniques such as radiolabel incorporation during chain extension are possible but it is preferred to use the lac operator sequence which is preferably incorporated

into the DNA during amplification, as discussed above. Full chain extension creates the double stranded DNA sequence which is bound by the lac I repressor-β galactosidase fusion protein. Bound fusion protein can then be identified colorimetrically as discussed above and this identifies the three aliquots which have been extended, thereby identifying the dideoxy base which was added in the remaining aliquot.

With regard to the non-extended DNA, where extension of the loop-attached 3' primer was blocked by a dideoxynucleotide, again a number of means for identification are possible and will be readily apparent to the skilled person. Preferably, a probe which hybridises downstream of the 3' end of the loop-attached primer is used, i.e. the probe hybridises to the immobilised strand between 5' end of immobilisation and the 3' loop structure. The probe is suitably labelled or provided with means for attaching a label. Such a probe will bind to the single strand DNA but will not bind to the double stranded DNA.

If desired, both double and single stranded DNA can be identified and this provides additional checking for the accuracy of the results. It will usually be desirable to run a control with no dideoxynucleotides and a 'zero control' containing a mixture of all four dideoxynucleotides.

Another means of identification is that disclosed in our co-pending application of even date (Agents ref.: 75.57799) which relates to detection of pyrophosphate released during chain extension. When each nucleotide is incorporated a pyrophosphate group is split off the nucleotide triphosphate and the remaining nucleotide monophosphate is incorporated at the end of the growing nucleic acid chain. In those aliquots which have not incorporated a chain terminating dideoxynucleotide there is extensive pyrophosphate release during chain extension. This release of pyrophosphate can be

measured using luciferin and luciferase which emit light in substantially direct proportion to the amount of pyrophosphate present.

In many diagnostic applications, for example genetic testing for carriers of inherited disease, the sample will contain heterozygous material, that is half the DNA will have one nucleotide at the target position and the other half will have another nucleotide. Thus of the four aliquots used in the method of the invention, two will show a positive signal and two will show half the positive signal. It will be seen therefore that it is desirable to quantitatively determine the amount of label detected in each sample. In the case of a homozygous sample it will be clear that there will be three negatives and one positive signal of the four aliquots.

The invention will now be described by way of a non-limiting example with reference to the drawings in which:

Fig.1 shows a protocol for identifying a base in a single target position using the method according to the invention;

Figs.2 and 3 show oligonucleotide primers used in Example 1; and

Fig.4 is a graph showing the results obtained in the Example.

MATERIALS AND METHODS

Bacterial strains and enzymes. Escherichia coli RRI ΔM15 (Rüther, U(1982), Nucl. Acids Res., 10 5765-5772) was used as bacterial host. The plasmid vector used was pRIT 28 (Hultman, T., Ståhl, S., Moks, T. and Uhlén, M. (1988) "Approaches to solid phase DNA sequencing", Nucleosides and Nucleotides 7 629-638). Restriction endonucleases, DNA polymerase I (Klenow Fragment), T7 DNA Polymerase, CIP and T4 polynucleotide Kinase were obtained from Pharmacia,

Sweden. The Taq DNA polymerase used was purchased from Perkin-Elmer, Ca., U.S.A. (AmpliTaq).

Example 1

Synthesis of oligonucleotides. 5 oligonucleotide primers (See figures) RIT 321, RIT 322, RIT 331, RIT 333 and RIT 338, complementary to regions encoding a part of the active site of the HIV reverse transcriptase gene (RT) (bases 625 to 1165 Myers, G., Korber, B., Berkovsky, J.A. Smith, R.F. and Pavlakis, G.N. Human Retroviruses and AIDS 1991 (Los Alamos National Laboratory, New Mexico 1991)), were synthesized by phosphoramidite chemistry on an automated DNA synthesis apparatus (Gene Assembler Plus, KABI-Pharmacia, Sweden) as described by the manufacturer. RIT322 was biotinylated by using a biotin phophoramidite (Clonetech, Ca, U.S.A.). Purification was performed on a pepRPC 5/5 reversed phase column (KABI-Pharmacia, Sweden).

PCR cloning

The HIV RT fragment was cloned by amplification from a clinical sample obtained from a patient with HIV-1 (Swedish Bacteriology Laboratory, SBL, Stockholm, Sweden) using 5 pmol each of the oligonucleotides RIT331 and RIT333 (figure 3) both containing "handles" in order to introduce an upstream Bam HI and a downstream Eco RI recognition sites. The PCR reaction mix contained 200 μM dNTPs, 20 mM Tris-HCl (pH 8.7), 2 mM $MgCl_2$, 0.1% Tween 20 and 0.5 units AmpliTaq resulting in a final volume of 50 μl. The temperature profile was set up by a denaturation step at 95°C for 0.5 min. followed by a primer annealing step at 55°C for 0.5 min. and a final extension step at 72°C for 2 mins. These steps were repeated 30 times using a Gene Amp PCR System, PE 9600

(Perkin Elmer, Ca., U.S.A.). The PCR amplified HIV RT fragment and the pRIT 28 vector were both restricted with Bam H1 and Eco R1, cut out and purified from agarose and then ligated for 1 hour in room temperature. The construction was transformed into competent RRIΔM15cells and spread on TBAB (Sambrook, J. et al loc. cit.) plates containing IPTG(n-isopropyl-β-D-thiogalactopyranoside) X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and ampicillin allowing blue/white selection (Langley, E.K. et al (1975) PNAS (USA) 72, 1254-1257). Five white colonies containing the plasmid with a correct insert was confirmed by solid phase sequencing (Hultman, T. et al (1991) Biotechniques 10, 84-93). One of those clones was designated pRIT-RT and choosen for further studies. This clone is stored at the Department of Biochemistry, Royal Institute of Technology, Stockholm, Sweden.

Template preparation for DIANA detected Mini Sequencing

A colony harbouring pRIT28-RT was transferred to a vial and lysed at 99°C for 5 min. in 10μl 20 mM Tris-HCl (pH 8.7). 1 μl lysate was subsequently transferred to a PCR mixture of 5 pmol RIT135 and RIT322 (biotinylated), 0.25 pmol RIT321, 200 μM dNTPs, 20 mM Tris-HCl (pH 8.7), 2 mM $MgCl_2$, 0.1% Tween 20 and 0.5 units AmpliTaq to a final volume of 50 μl. It will be noted that primer RIT322 comprises a 5' Biotin, for subsequent attachment to a streptavidin coated solid support, and the 21 bases which define the lac Op recognition sequence. Amplification was performed as above and the resulting PCR product was subsequently immobilized (Hultman, T. et al (1989) Nuc. Acids Res. 17, 4937-4946) on prewashed streptavidin coated paramagnetic beads, (Lea, T. et al (1988) J. Mol. Recognit 1, 9-18) Dynabeads M280-Streptavidin (Dynal AS, Norway), prewashed with binding solution according to manufacturer. After

immobilization, the beads were rinsed with 50 µl binding-washing solution and assayed for bound DNA. The beads with the immobilized DNA were mixed with 50 µl of the fusion protein, lacI-β-galactosidase (Dynal AS, Norway), and incubated for 20 minutes. Excess of the fusion protein was removed by washing the beads 4 times with DIANA buffer (Dynal AS, Norway) and changing to new tubes in the last step in order to avoid background due to coating of the walls. 100 µl of chromogenic substrate, ortho-nitrophenyl-β-D-galactoside (ONPG, 1.25 mg/ml), was added and after 6 min. the reaction was stopped by an addition of 100 µl 1M Na_2CO_3 and the supernatant was analyzed in an EAR340AT ELISA plate reader (SLT-Labinstruments, Austria) by measuring the absorbence at 405 nm. The strands were separated by melting by incubation with 20 µl 0.1 M NaOH for 5 min. generating single stranded immobilized DNA template, which was once again washed with 50 µl binding solution, 50 µl 1 x TE.

Mini Sequencing reactions

Six separate extension reactions with respect to the appropriate dideoxy nucleotide were set up (one with only ddATP, one with only ddCTP, one with only ddGTP, one with only ddTTP, one with all four ddNTPS present and one without any of ddNTPs) in a total of 10 µl containing 2 µl of the annealing mixture, 17 mM Tris-HCl (pH7.5), 6 mM $MgCl_2$, 1 mM DTT, 1 µM of the appropriate dideoxy nucleotide and 0.13 units of Sequenase ver. 2. A schematic outline of the experiment is shown in figure 1. The dideoxy incorporation was performed at room temperature for 5 mins. and stopped by adding 20 µl 0.5M EDTA. Thereafter the beads were washed twice with 30 µl 10 mM Tris-HCl (pH 7.5). In the following extension step 200 µM dNTP concentration was used together with 25 mM Tris-HCl (pH 7.5), 12.5 mM $MgCl_2$, 1 mM DDT and 0.13

units Sequenase in a total of 10 µl. In the aliquots where a dideoxy nucleotide had not been incorporated, the Sequenase leads to a chain extension and to full double stranded DNA being attached to the beads. After a 5 min. incubation in room temperature 20 µl 0.5 M EDTA was added and the beads were washed with 40 µl DIANA buffer (Dynal AS. Norway) (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% Tween 20, 1 mM $MgCl_2$ and 10 mM β-mercaptoethanol).

Detection by DIANA

The results were detected by DIANA (Wahlberg, J., Lundeberg, J., Hultman, T. and Uhlén, M. (1990) "General colorimetric method for DNA diagnostics allowing direct solid-phase genomic sequencing of the positive samples." Proc. Natl. Acad. Sci U.S.A. 87, 6569-6573). The beads with the immobilized DNA were mixed with 50 µl of the fusion protein, lacI-β-galactosidase (Dynal AS, Norway), and incubated for 20 minutes. Excess of the fusion protein was removed by washing the beads 4 times with DIANA buffer (Dynal AS, Norway) and changing to new tubes in the last step in order to avoid background due to coating of the walls. 100 µl of chromogenic substrate, ortho-nitrophenyl-β-D-galactoside (ONPG, 1.25 mg/ml), was added and after 6 min. the reaction was stopped by an addition of 100 µl 1M Na_2CO_3 and the supernatant was analyzed in an EAR340AT ELISA plate reader (SLT-Labinstruments, Austria) by measuring the absorbence at 405 nm. The results are shown in figure 4. The assay show that a low signal is obtained when all four dideoxynucleotides (ddNTP) are used as well as when only ddATP is used. Since the complementary base next to the 3'-end of the sequencing primer is a dideoxythymidine, the result demonstrates that the assay can be used to detect a base sequence at a specific point.

Claims

1. A method of introducing a 3'-terminal loop structure onto a target sequence of one strand of double stranded DNA, said target sequence having a region A at the 3'-terminus thereof and there being optionally a DNA region B which extends 3' from region A, whereby said double-stranded DNA is subjected to polymerase chain reaction (PCR) amplification using a first primer hybridising to the 3'-terminus of the sequence complementary to the target sequence, which first primer is immobilised or provided with means for attachment to a solid support, and a second primer having a 3'-terminal sequence which hybridises to at least a portion of A and/or B of the target sequence while having at its 5'-end a sequence substantially identical to A, said amplification producing double-stranded target DNA having at the 3'-end of the target sequence, in the following order, the region A, a region capable of forming a loop and a sequence A' complementary to sequence A, whereafter the amplified double-stranded DNA is subjected in immobilised form to strand separation whereby the non-immobilised target strand is liberated and region A' is permitted or caused to hybridise to region A, thereby forming said loop.

2. A method as claimed in claim 1 in which after final strand separation of the amplified double stranded DNA, the region A' on the immobilised single stranded DNA strand is caused to hybridise to the region A by annealing at a temperature above the self-annealing temperature of the second primer.

3. A method as claimed in claim 1 or claim 2 in which the immobilised single stranded DNA after loop formation contains a restriction site in the double stranded region adjacent to the loop.

4. A method as claimed in any of the preceding claims in which the first primer carries biotin as means for

immobilisation.

5. A method as claimed in any of the preceding claims in which the first primer carries a recognition site for a DNA binding protein carrying a label.

6. A method as claimed in any of the preceding claims in which the immobilised single stranded DNA after loop formation is subjected to sequencing using the 3'-end of the A' sequence as the primer for sequencing.

7. A method as claimed in claim 6 in which the region A' is chosen to hybridise immediately adjacent to a single target base to be determined in the target sequence and the immobilised single stranded DNA is subjected to a chain extension reaction in the presence of a specific deoxynucleoside or dideoxynucleoside whereby chain extension or absence of chain extension provides an indication of the presence or absence of said target base.

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FIG.1

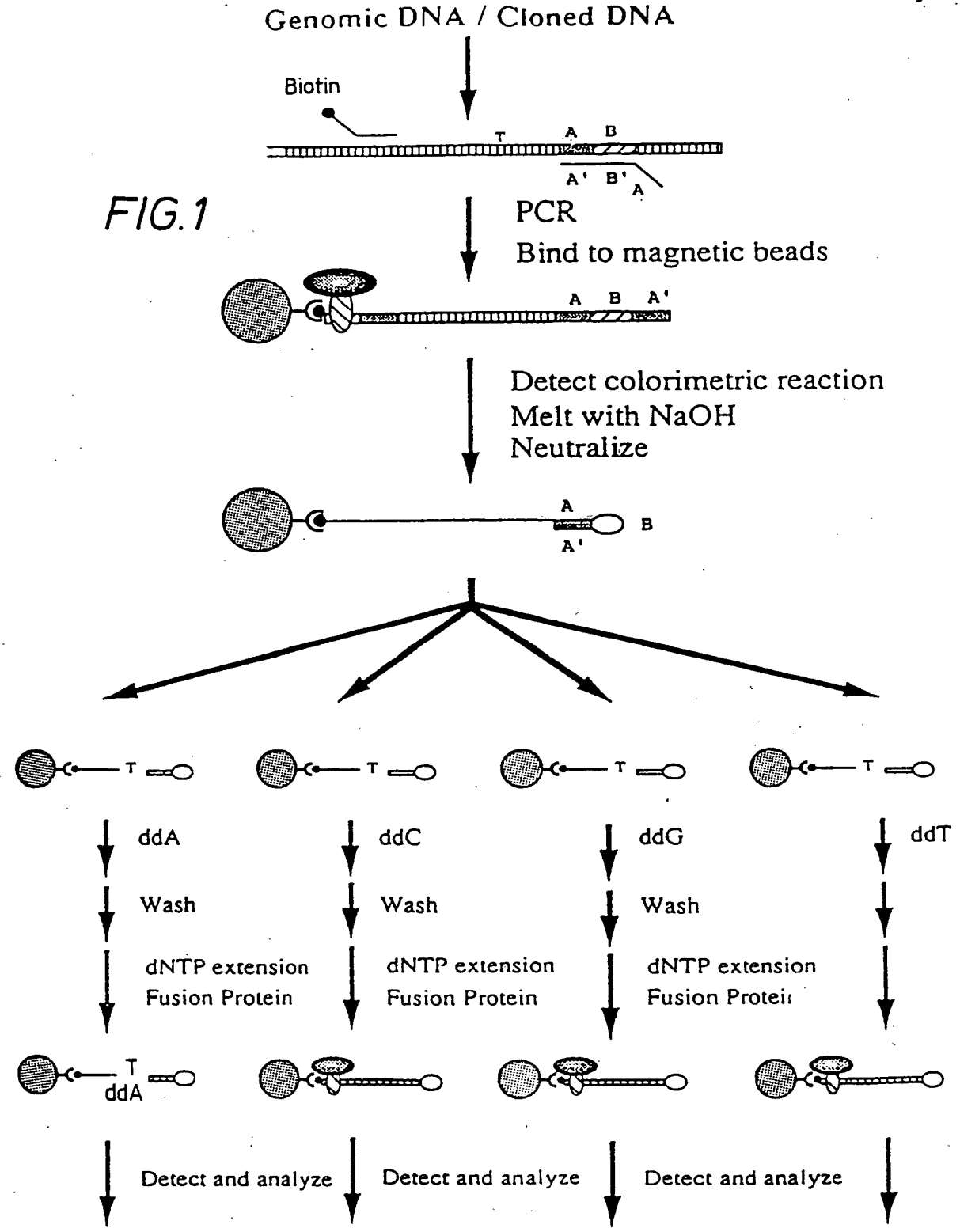
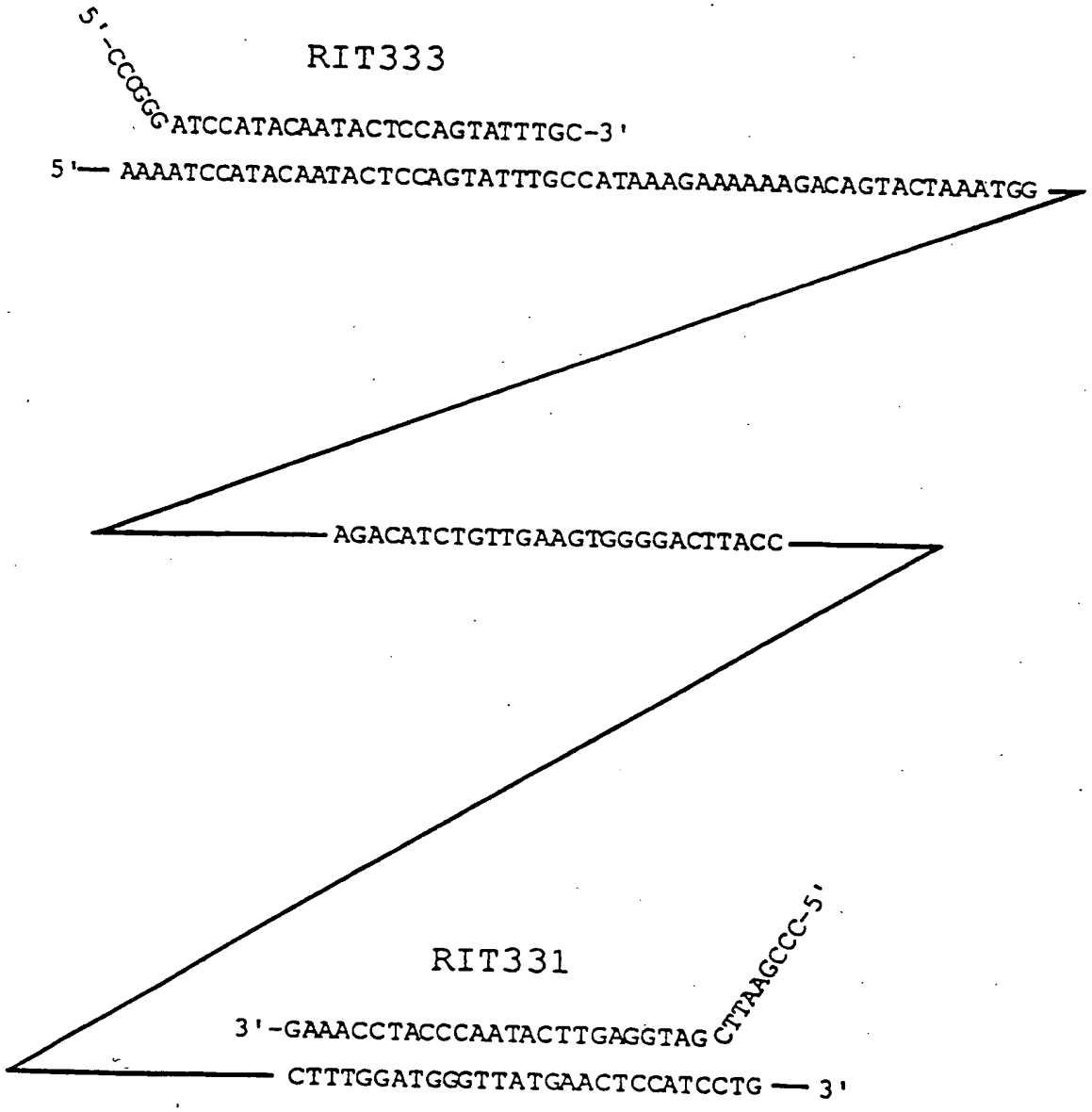

Genomic DNA / Cloned DNA
Biotin
T
A B
A' B' A
PCR
Bind to magnetic beads
A B A'
Detect colorimetric reaction
Melt with NaOH
Neutralize
A
B
A'
T
ddA
ddC
ddG
ddT
Wash
dNTP extension
Fusion Protein
Detect and analyze

FIG. 2


RIT333
5'-CCGGG
ATCCATACAATACTCCAGTATTTGC-3'
5'— AAAATCCATACAATACTCCAGTATTTGCCATAAAGAAAAAAGACAGTACTAAATGG —
AGACATCTGTTGAAGTGGGGACTTACC
RIT331
3'-GAAACCTACCCAATACTTGAGGTAG
CTTAAGCCC-5'
CTTTGGATGGGTTATGAACTCCATCCTG — 3'

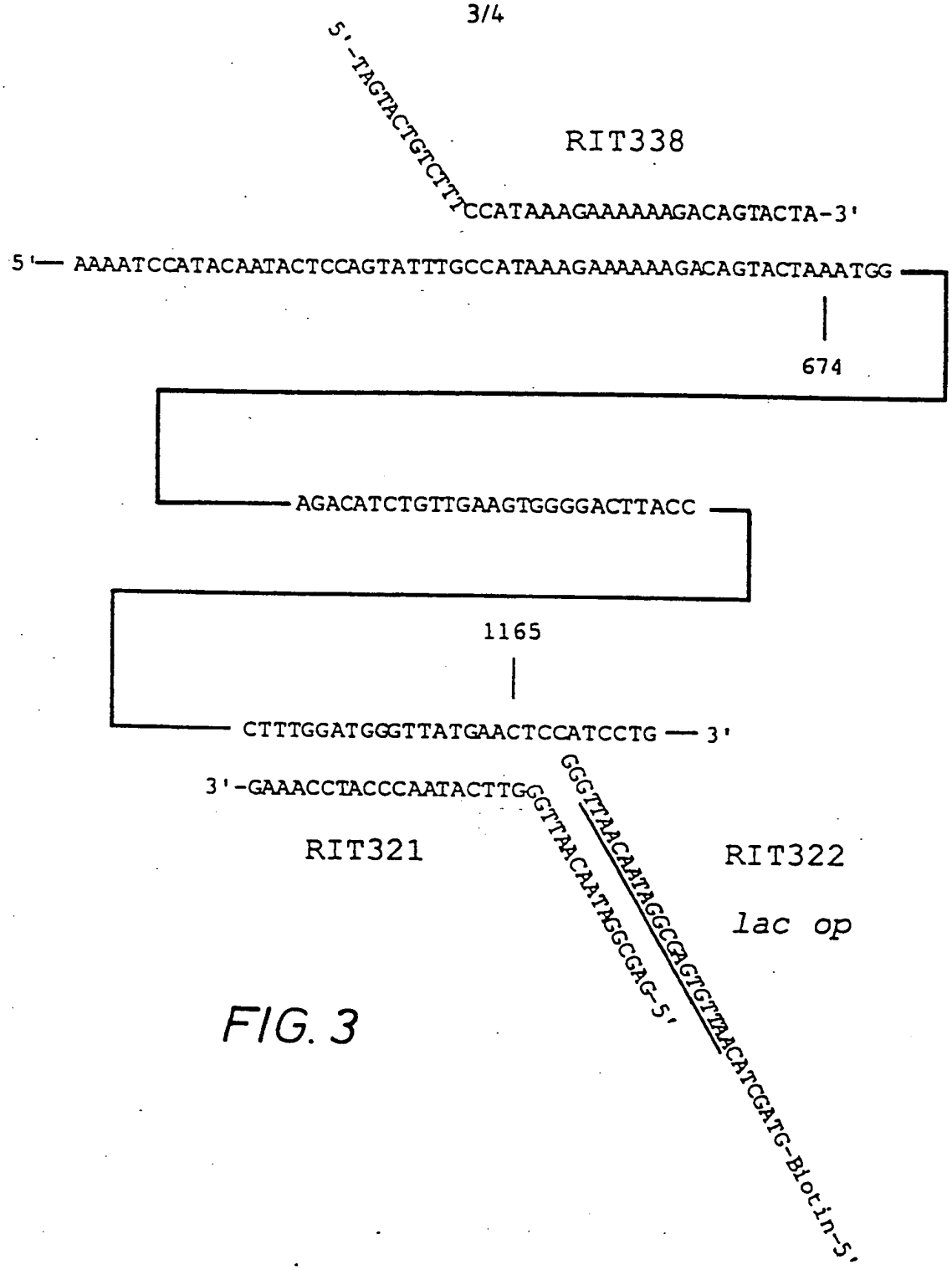
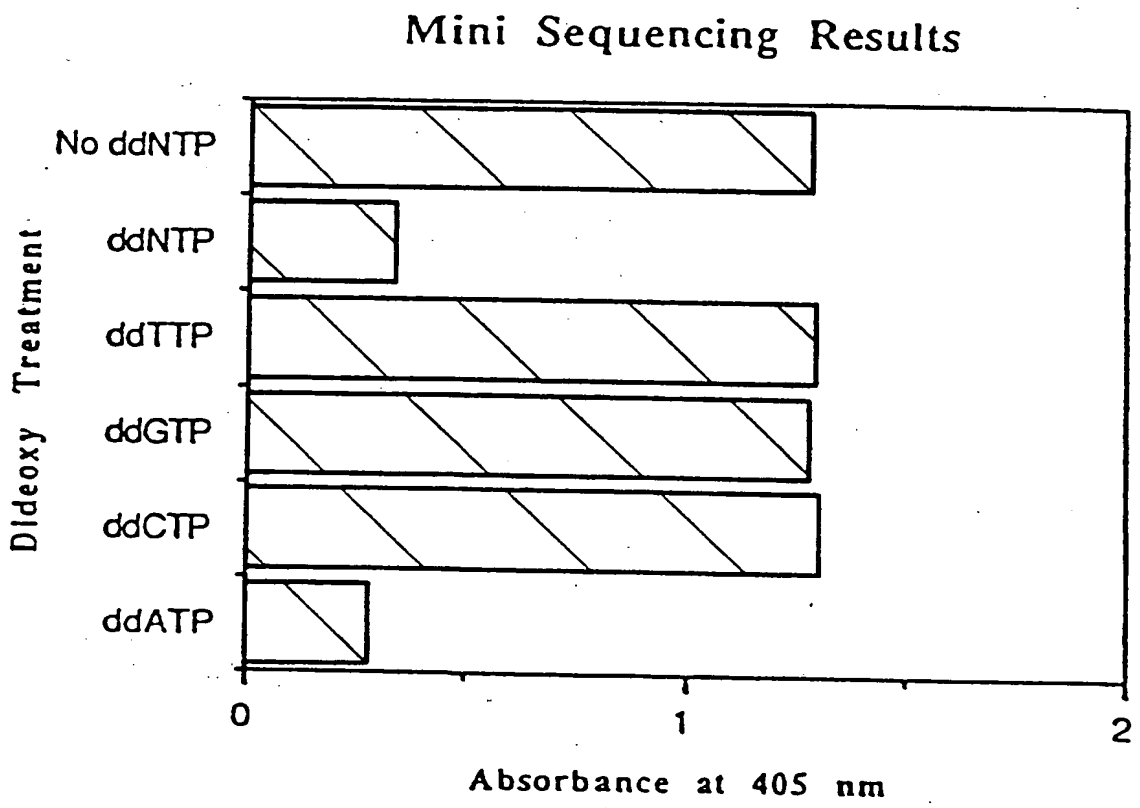

5'-TAGTACTGTCTTTCCATAAAGAAAAAAGACAGTACTA-3'
RIT338
5'— AAAATCCATACAATACTCCAGTATTTGCCATAAAGAAAAAAGACAGTACTAAATGG
674
AGACATCTGTTGAAGTGGGGACTTACC
1165
CTTTGGATGGGTTATGAACTCCATCCTG — 3'
3'-GAAACCTACCCAATACTTGGGTTAACAATAGGCGAG-5'
GGGTTAACAATAGGCGAGTGTTAACATCGATG-Biotin-5'
RIT321
RIT322
lac op

FIG. 3

FIG. 4


Mini Sequencing Results
No ddNTP
ddNTP
ddTTP
ddGTP
ddCTP
ddATP
Dideoxy Treatment
0
1
2
Absorbance at 405 nm

IN NATIONAL SEARCH REPORT

International Application No PCT/EP 93/01204

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	EP,A,0 469 755 (SYNTEX) 5 February 1992 see page 3, line 57 - page 5, line 10 see page 12, line 29 - line 49 see page 13, line 17 - line 54 see page 19, line 12 - line 39; claims	1
P,A	EP,A,0 530 112 (UNIVERSITY OF MEDICINE AND DENTISTRY OF NEW JERSEY) 3 March 1993 see page 2, line 40 - page 3, line 41; claims	1

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26 AUGUST 1993	06. 09. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LUZZATTO E.R.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9301204
SA 74814

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0469755	05-02-92	CA-A- 2047342	20-01-92
		JP-A- 5084079	06-04-93
EP-A-0530112	03-03-93	CA-A- 2073630	01-03-93